Doc2b Is a Key Effector of Insulin Secretion and Skeletal Muscle Insulin Sensitivity

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Exocytosis of intracellular vesicles, such as insulin granules, is carried out by soluble N-Ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Sec1/Munc18 (SM) proteins. An additional regulatory protein, Doc2b (double C2 domain), has recently been implicated in exocytosis from clonal β-cells and 3T3-L1 adipocytes. Here, we investigated the role of Doc2b in insulin secretion, insulin sensitivity, and the maintenance of whole-body glucose homeostasis. Doc2b heterozygous (Doc2b+/−) and homozygous (Doc2b−/−) knockout mice exhibited significant whole-body glucose intolerance and peripheral insulin resistance, compared with wild-type littermates. Correspondingly, Doc2b+/− and Doc2b−/− mice exhibited decreased responsiveness of pancreatic islets to glucose in vivo, with significant attenuation of both phases of insulin secretion ex vivo. Peripheral insulin resistance correlated with ablated insulin-stimulated glucose uptake and GLUT4 vesicle translocation in skeletal muscle from Doc2b−/− mice, which was coupled to impairments in Munc18c-syntaxin 4 dissociation and in SNARE complex assembly. Hence, Doc2b is a key positive regulator of Munc18c-syntaxin 4-mediated insulin secretion as well as of insulin responsiveness in skeletal muscle, and thus a key effector for glucose homeostasis in vivo. Doc2b’s actions in glucose homeostasis may be related to its ability to bind Munc18c and/or directly promote fusion of insulin granules and GLUT4 vesicles in a stimulus-dependent manner.

Glucose homeostasis is maintained through the coordinated actions of insulin secretion and insulin action; dysfunction of insulin action yields insulin resistance and, when coupled with dysfunctional insulin secretion, results in type 2 diabetes. Common to both insulin action and insulin secretion mechanisms is the requirement for SNARE protein–regulated vesicle/granule exocytosis. Two target membrane SNAREs (t-SNAREs) and one vesicle/granule membrane SNARE (v-SNARE) combine to form a heterotrimERIC SNARE core complex that drives vesicle fusion in the exocytosis process. Insulin-containing secretory granules within the islet β-cell contain the v-SNARE vesicle-associated membrane protein 2 (VAMP2), which assembles at the plasma membrane (PM) with either the t-SNARE combination syntaxin 1A and synaptosome-associated protein of 25 kDa (SNAP25), syntaxin 4 and SNAP25, or syntaxin 4 and SNAP23 to release insulin (1,2). In a similar fashion, insulin promotes glucose uptake into peripheral skeletal muscle and adipose tissues by translocation and fusion of the GLUT4 glucose transporter vesicles to the cell surface (3,4). Insulin action requires only the t-SNAREs syntaxin 4 and SNAP23 and the v-SNARE VAMP2 (5), all of which are also required for insulin secretion from the β-cell. Defects and/or deficiencies of these SNARE proteins are associated with insulin resistance and/or insulin insufficiency in type 2 diabetes (6–9).

Insulin secretion and GLUT4 recruitment events are highly regulated, maintained at very low levels in the absence of appropriate stimuli, and rapidly and robustly activated in response to stimuli. Maintenance and activation of exocytotic processes are regulated by the Sec1/Munc18 (SM) proteins, which bind and facilitate the accessibility of syntaxin to interact with its cognate SNARE partners (10,11). SM-syntaxin proteins pair in a 1:1 stoichiometry and in multiple modes. In one state, syntaxin is kept in a "closed" conformation, preventing its participation in the SNARE core complex (12). Upon stimulation, the SM protein is presumed to dissociate or reposition syntaxin so that it “opens” and is accessible to SNARE complex assembly. In homogenates of β-cells, adipocytes, and skeletal muscle, Munc18c binds to syntaxin 4 in the absence of stimuli, dissociating in response to stimuli while undergoing tyrosine phosphorylation (13–15). We recently identified the insulin receptor to serve as a Munc18c tyrosine kinase in 3T3-L1 adipocytes and skeletal muscle (13).

Concurrent with its dissociation from syntaxin 4, phosphorylated Munc18c switches its affinity toward binding to the protein Doc2b (double C2 domain) in MIN6 clonal β-cells (16). Elevation of calcium is reported to yield complexation of Doc2b with syntaxin 4 as well (17). Doc2b is a member of the double C2 domain protein family, containing two calcium and phospholipid binding domains in its COOH terminus. In vitro, Doc2b selectively binds to Munc18-1 via Doc2b domain C2A and binds to Munc18c via Doc2b domain C2B (18,19). In MIN6 β-cell and 3T3-L1 adipocyte clonal cell studies, the inhibitory RNA–mediated reduction of Doc2b attenuates stimulus-induced insulin exocytosis and GLUT4 exocytosis events, respectively (17,18,20). By contrast, Doc2b overexpression in these cell types enhances stimulus-induced exocytosis but not basal exocytosis (17,18,20). Unlike Munc18c and syntaxin 4, the relevance of Doc2b function for whole-body glucose homeostasis remains untested. Moreover, although Doc2b mRNA abundance in islets of obese mice was significantly reduced (8), Doc2b deficiency has yet to be correlated with type 2 diabetes, such that its promise as a novel therapeutic target remains in question.

In this study, we used classic Doc2b knockout mice to investigate the role of Doc2b in insulin granule exocytosis
and insulin-stimulated GLUT4 vesicle translocation, culminating in a new in vivo model of glucose intolerance and insulin resistance. Mechanistically, islet perfusion studies revealed Doc2b to function in both phases of glucose-stimulated insulin secretion, implicating Doc2b to act on both types of SNARE complexes. Furthermore, skeletal muscle fractionation studies demonstrated a requirement for Doc2b in insulin-stimulated GLUT4 accumulation to mediate glucose uptake; SM-SNARE interactions in muscle fractions were found to be altered by Doc2b deletion.

**RESULTS**

**Doc2b knockout mice are glucose intolerant.** Although the Doc2b−/− mice have been characterized for alterations in neuronal protein expression and synaptic vesicle trafficking function, such studies are lacking in evaluation of Doc2b function in tissues relevant to glucose homeostasis. Gene ablation in the heterozygous and homozygous knockout mice was confirmed by comparing Doc2b mRNA levels in Doc2b−/+ (wild type [WT]) littersmates in brain, skeletal muscle (whole hindlimb), liver, and fat (epididymal) by Q-PCR (Fig. 1A). Protein levels of Doc2b were reduced, in the absence of significant differences in other SNARE or SM proteins implicated in insulin exocytosis, in islets isolated from Doc2b−/− and Doc2b−/− knockout mice, compared with the Doc2b+/+ islets (Fig. 1B and Supplementary Fig. 1). The Doc2b antibody showed nonspecific background, consistent with previous work (23). Doc2b levels were reduced in heart, skeletal muscle, liver, and fat tissues of Doc2b−/− and Doc2b−/− mice, without alternations in abundance of syntaxin 4, SNAP23, VAMP2, or Munc18c (Fig. 1C). Abundance of the glucose transporter GLUT4 protein was also unchanged in heart, skeletal muscle, and fat from WT or Doc2b-deficient mice (Supplementary Fig. 1).

To determine the effects of Doc2b deficiency upon whole-body glucose tolerance, 4–6-month-old Doc2b+/+, Doc2b−/−, and Doc2b−/− mice (4–6 months old) were fasted for either 6 h (0800–1400 h) or 12 h (1400–0000 h) before intraperitoneal glucose tolerance test (IPGTT), as specified in the figure legend. After sample collection of fasted blood, animals were injected intraperitoneally with 10 units/kg insulin or saline buffer for 30 min, and perfused at a rate of 0.3 mL/min during stimulations with 20 mmol/L glucose and 35 mmol/L KCl, with fractions collected every 1–5 min for quantitation by radioimmunoassay (Millipore).

**Islet RNA isolation and quantitative PCR.** Total RNA from mouse islets were isolated using the RNAeasy mini kit (Qiagen). RNA (2 μg) was reverse transcribed with the SuperScript First Strand cDNA Synthesis Kit (Invitrogen), and 1% of the product was used for quantitative PCR (Q-PCR). The primers used were as follows: Doc2b primers, forward 5′-ccagcaaggcaataagctc and reverse 5′-ccagcaaggcaataagctc; and GAPDH primers, forward 5′-tttgggatgtagttgcccttt and reverse 5′-tttgggatgtagttgcccttt. The Q-PCR conditions were as follows: 95°C for 2 min hold (UD6 incubation), 95°C for 2 min hold, 40 cycles of 95°C for 15 s, and 60°C for 30 s.

**Statistical analysis.** All data were evaluated for statistical significance using Student’s t test for pairwise comparison of two groups (i.e., Doc2b+/+ or Doc2b−/−, or Doc2b+/+ vs. Doc2b−/−). Data are expressed as the mean ± SE.
isolated islets from male Doc2b+/+, Doc2b+/−, and Doc2b−/− mice for perfusion analyses. Ex vivo, insulin secretion under basal conditions was similar among all three islet groups (Fig. 3A), similar to our findings of insulin content in fasted serum. Glucose stimulation (20 mmol/L) elicited a 12-fold peak increase in insulin release from WT islets during the initial phase, whereas Doc2b−/− and Doc2b−/− islets showed less response. During the second phase, Doc2b−/− and Doc2b−/− islets secreted substantially less insulin (Fig. 3B). Consistent with impaired first-phase glucose-stimulated insulin secretion, KCl-stimulated insulin release was precipitously decreased as Doc2b expression decreased (Fig. 3C). Insulin content in Doc2b−/− and Doc2b−/− islets was comparable to that in WT islets (Fig. 3D). These data indicated that Doc2b-depleted islets lacked function during both phases of glucose-stimulated insulin secretion, corroborating the deficient serum insulin content observed during the IPGTT in the Doc2b−/− mice. This is the first demonstration of a Doc2b requirement in both phases of insulin secretion from islets.

**Impaired insulin sensitivity, skeletal muscle glucose uptake, and GLUT4 translocation in Doc2b knockout mice.** Whole-body glucose intolerance could also be attributable to defects in insulin sensitivity, causing insulin resistance. To investigate this, 4- to 6-month-old Doc2b+/+, Doc2b+/−, and Doc2b−/− male mice were subjected to an ITT. As expected of WT mice of this age and strain, insulin injection resulted in a sharp ~45% decline in blood glucose within 60 min (Fig. 4A). By contrast, neither Doc2b+/+ nor Doc2b−/− mice dropped below 70% of starting glucose levels, with levels back on the rise by 60 min postinjection. Analysis of AUC revealed a substantial difference in glucose levels during the ITT (in arbitrary units) WT = 5,971 ± 238, Doc2b+/+ = 7,019 ± 420, and Doc2b−/− = 7,210 ± 420), implicating a defect in the peripheral glucose uptake resulting from Doc2b depletion.

Skeletal muscle GLUT4-mediated glucose uptake accounts for ~80% of whole-body glucose clearance, and so largely controls the response in the ITT (28). To assess insulin-stimulated GLUT4 translocation in skeletal muscle, sarcolemma/transverse tubule–enriched fractions (referred to as P2 fractions) were prepared from insulin- or saline-injected mice as described previously (24,25,29,30). A statistically significant, nearly twofold increase in GLUT4 accumulation into the P2 membrane fraction was detected from insulin-stimulated WT mouse muscle (Fig. 4B). Remarkably, no insulin-stimulated increase in GLUT4 accumulation was observed in Doc2b−/− mice. P2 fractions prepared from unstimulated WT and Doc2b−/− mice showed similarly low levels of GLUT4 protein. Consistent with this, EDL muscle from Doc2b−/− mice showed a lack of insulin-stimulated 3H-2-deoxyglucose uptake, in contrast to the nearly twofold increase seen in WT EDL muscle (Fig. 4C). Proximal insulin signaling in skeletal muscle and liver was unaffected, as determined by insulin-stimulated AKT and AKT phosphorylation and equivalent AKT expression (Fig. 4D). Taken together, these data demonstrate that insulin-stimulated GLUT4 externalization and glucose uptake is significantly impaired in skeletal muscle tissue of Doc2b−/− mice.

**Altered SM and SNARE complex formations in skeletal muscle of the Doc2b knockout mice.** To date, all studies regarding the mechanistic role of Doc2b are from in vitro and cell culture model systems, and results are controversial due to methodological differences (17,18,20). To resolve these issues, we tested previously described Doc2b interactions...
using skeletal muscle of insulin-injected mice as a more physiologically relevant model system. Because calcium has been shown to trigger Doc2b association with syntaxin 4 in vitro (17), we examined binding under calcium-deficient (2 mmol/L EDTA) and calcium-supplemented (1 mmol/L CaCl$_2$) conditions. In WT muscle lysates, Doc2b binding to Munc18c increased by ~60% in response to insulin stimulation; calcium addition to the lysis buffer failed to significantly alter either basal or insulin-stimulated binding events (Fig. 5A). Similar results were obtained using basal or glucose-stimulated MIN6 cell lysates supplemented with calcium in the lysis buffer (Supplementary Fig. 3). In skeletal muscle lysates, anti-Munc18c coprecipitated syntaxin 4 regardless of calcium supplementation, whereas neither VAMP2 nor SNAP23 coprecipitated with Munc18c under any conditions (Fig. 5B). Reciprocal anti-VAMP2 immunoprecipitation reactions showed no binding of Munc18c. Calcium supplementation did not impact SNARE complex formation; ratios of SNAP23/VAMP2 and syntaxin 4/VAMP2 normalized to 1.0 in the absence of calcium (2 mmol/L EDTA), were measured to be 0.8 ± 0.2 and 0.8 ± 0.2, respectively, in the presence of supplemental calcium (n = 3 paired experiments, P > 0.05). Moreover, syntaxin 4 failed to coprecipitate with Doc2b, even under calcium-containing and insulin-stimulated conditions from skeletal muscle (Fig. 5C); Doc2b$^{-/-}$ muscle served as control for nonspecific binding. Syntaxin 4 coprecipitated SNAP23 equivalently under all conditions, consistent with SNAP23 participation in binary and ternary SNARE complexes. Like syntaxin 4, which was constitutively present in the P2 fraction (Fig. 5D), Doc2b abundance was unchanged by insulin in P2 fractions prepared 5 min post–insulin injection, the time of peak tyrosine phosphorylation of Munc18c and its association with Doc2b, and Doc2b translocation was not detected within 30 min post–insulin injection (data not shown). These data suggest that in skeletal muscle lysate, Doc2b binds to Munc18c in an insulin-sensitive manner and fails to bind to syntaxin 4 in response to insulin and/or added calcium.

We next sought to determine why GLUT4 accumulation in the target membranes of skeletal muscle was impaired in the Doc2b$^{-/-}$ mice by examining effects upon SM and

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Doc2b$^{+/+}$</th>
<th>Doc2b$^{+/-}$</th>
<th>Doc2b$^{-/-}$</th>
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<tr>
<td>Glucose (mg/dL)</td>
<td>112 ± 9</td>
<td>121 ± 5</td>
<td>105 ± 2</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>96.8 ± 15.6</td>
<td>107.2 ± 13.2</td>
<td>89.1 ± 9.3</td>
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<tr>
<td>Cholesterol (mg/dL)</td>
<td>123.2 ± 6.5</td>
<td>118.2 ± 8.9</td>
<td>128.5 ± 5.1</td>
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<tr>
<td>NEFA (mmol/L)</td>
<td>1.19 ± 0.11</td>
<td>1.33 ± 0.12</td>
<td>1.32 ± 0.04</td>
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</tbody>
</table>

Data represent the average ± SE. No significant differences were detected. Serum was collected from 18-h fasted Doc2b$^{+/+}$, Doc2b$^{+/-}$, and Doc2b$^{-/-}$ male littermate mice at 4–6 months of age (n = 7 for each genotype) for determination of parameters shown.

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**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Doc2b$^{+/+}$</th>
<th>Doc2b$^{+/-}$</th>
<th>Doc2b$^{-/-}$</th>
</tr>
</thead>
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<tr>
<td>Body weight (g)</td>
<td>28.3 ± 0.8</td>
<td>30.7 ± 0.9</td>
<td>30.7 ± 0.8</td>
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<tr>
<td>Tissue (% body weight)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Liver</td>
<td>3.61 ± 0.23</td>
<td>2.85 ± 0.23</td>
<td>3.92 ± 0.10</td>
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<tr>
<td>Lung</td>
<td>0.65 ± 0.11</td>
<td>0.82 ± 0.08</td>
<td>0.81 ± 0.09</td>
</tr>
<tr>
<td>Heart</td>
<td>0.62 ± 0.18</td>
<td>0.79 ± 0.20</td>
<td>0.91 ± 0.07</td>
</tr>
<tr>
<td>Fat</td>
<td>2.66 ± 0.53</td>
<td>2.24 ± 0.49</td>
<td>2.20 ± 0.23</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.77 ± 0.11</td>
<td>0.96 ± 0.05</td>
<td>1.04 ± 0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.32 ± 0.07</td>
<td>1.17 ± 0.05</td>
<td>1.36 ± 0.10</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.52 ± 0.14</td>
<td>1.22 ± 0.12</td>
<td>1.57 ± 0.12</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.09 ± 0.48</td>
<td>0.99 ± 0.42</td>
<td>1.03 ± 0.23</td>
</tr>
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</table>

Data represent the average ± SE. Weights were collected from Doc2b$^{+/+}$, Doc2b$^{+/-}$, and Doc2b$^{-/-}$ male littermate mice at 4–6 months of age (n = 7 for Doc2b$^{+/-}$ and Doc2b$^{-/-}$; n = 5 for Doc2b$^{+/+}$) for determination of parameters shown. No statistical differences were detected.
Ablation of insulin-stimulated GLUT4 vesicle translocation in skeletal muscle. The mechanism(s) of Doc2b-dependent insulin granule secretion from pancreatic islets is a topic of great interest in diabetes susceptibility. Here, we present the first time that Doc2b is a key effector for insulin-stimulated GLUT4 vesicle translocation in skeletal muscle, and for both phases of glucose-stimulated insulin secretion from pancreatic islets. Doc2b associates with Munc18c in an insulin-dependent manner, but Doc2b binding to syntaxin 4 was not detected. Notably, Munc18c–syntaxin 4 association was increased in the absence of Doc2b, suggesting that this increased association is inhibitory for the insulin-stimulated, syntaxin 4–mediated docking/fusion of GLUT4 vesicles. Strikingly, the disease phenotype of the Doc2b<sup>−/−</sup> knockout mouse was almost as severe as that of the Doc2b<sup>−/−</sup> mouse, suggesting that Doc2b haploinsufficiency is worthy of future investigation in diabetes susceptibility. Mechanism(s) of Doc2b-dependent insulin granule and GLUT4 vesicle fusion events. Unlike other secretory cell types, islet β-cells require multiple Munc18 and syntaxin isoforms, otherwise sharing SNAP25/SNAP23 and VAMP2, for two distinct phases of glucose-stimulated insulin secretion. Syntaxin IA<sup>−/−</sup> null islets lack first-phase insulin release, whereas Munc18c and syntaxin 4 are imperative for second-phase insulin release from islets (27,31,32); Munc18-1 null islet perfusion has yet to be reported, although Munc18-1 and Munc18-2 were recently implicated in fast calcium–dependent exocytosis in electrophysiological studies (33). Demonstrating here that Doc2b is required for both phases of insulin release from primary islets, we speculate that Doc2b is a key effector for insulin-stimulated GLUT4 vesicle translocation in skeletal muscle, and for both phases of glucose-stimulated insulin secretion from pancreatic islets. Doc2b associates with Munc18c in an insulin-dependent manner, but Doc2b binding to syntaxin 4 was not detected. Notably, Munc18c–syntaxin 4 association was increased in the absence of Doc2b, suggesting that this increased association is inhibitory for the insulin-stimulated, syntaxin 4–mediated docking/fusion of GLUT4 vesicles. Strikingly, the disease phenotype of the Doc2b<sup>−/−</sup> knockout mouse was almost as severe as that of the Doc2b<sup>−/−</sup> mouse, suggesting that Doc2b haploinsufficiency is worthy of future investigation in diabetes susceptibility.
FIG. 4. Impaired insulin sensitivity in Doc2b-deficient mice is coupled to impaired insulin-stimulated GLUT4 translocation in skeletal muscle. A: Insulin tolerance testing (ITT) of Doc2b+/+, Doc2b−/−, and littermate Doc2b+/+ male mice (seven sets of mice) was performed by intraperitoneal injection of insulin (0.75 units/kg of body weight) into 4–6-month-old male mice fasted for 6 h. Blood glucose was monitored before and at 15, 30, 60, and 90 min after injection as described in RESEARCH DESIGN AND METHODS. Data shown are presented as mean percent of basal blood glucose concentration ± SE. *P < 0.05 vs. WT mice. B: Littermate sets of male WT or Doc2b−/− mice were fasted for 16 h and either left untreated or were injected with 21 units/kg body weight of insulin as described in RESEARCH DESIGN AND METHODS. Hindquarter muscles were homogenized and centrifuged to partition muscle into sarcolemmal/transverse tubule membrane and intracellular vesicular fractions. Proteins were resolved using SDS-PAGE for immunoblotting for GLUT4 (Ponceau S staining shows protein loading). Optical density quantitation of GLUT4 bands in three independent translocation assays is shown in the bar graph. *P < 0.05 compared with basal WT; **P < 0.05 compared with insulin-stimulated WT. C: In vitro 3H-2-deoxyglucose (2DG) uptake assay from EDL muscle of six pairs of WT and Doc2b−/− male mice (for each mouse, one muscle was left in the basal state and one was treated with insulin). *P < 0.05 compared with basal WT; **P < 0.05 compared with insulin-stimulated WT. D: Skeletal muscle and liver homogenates were prepared from mice stimulated with or without insulin and proteins were resolved on 10% SDS-PAGE for immunoblotting analysis of AKT activation assessed by anti-phospho-AKT (S473) immunoblotting. Blots were stripped and reprobed for total AKT content. Data are representative of three independent sets of tissue homogenates. IB, immunoblot.

that Doc2b regulates both Munc18-1-syntaxin 1A– as well as Munc18c-syntaxin 4–dependent secretion mechanisms. The role of Doc2b in the first phase went undetected in static incubation studies using stable Doc2b short hairpin RNA clonal β-cells (20) but is consistent with its role in Munc18c–syntaxin 1–driven exocytosis mechanisms in brain (23). The partial reduction of Doc2b in clonal β-cells may not have been sufficient to uncover the requirement for Doc2b in the first phase. Doc2b−/− islets retained >60% of first-phase function (while losing ~75% of second phase), and total ablation of Doc2b was required to detect a >50% loss of first-phase function. Our data does confirm the late-phase deficit reported in stable Doc2b short hairpin RNA clonal β-cells (20). Strikingly, second-phase secretion was nearly abolished in Doc2b−/− islets. Although our MIN6 β-cell studies support a mechanistic regulation of Munc18c–syntaxin 4 and SNARE complexes analogous to our studies with these proteins in skeletal muscle, future β-cell studies assessing the impact of Doc2b upon Munc18c− or −2 with syntaxin 1A are required, as well as assessment of all isoform-binding interactions in primary β-cells.

Doc2b is present in skeletal muscle transverse tubule/sarcotubular enriched subcellular fractions under basal conditions, and does not translocate, in contrast to GLUT4, in response to insulin stimulation. This finding is consistent with similar observations in glucose-stimulated MIN6 β-cells, yet counter to calcium-stimulated translocation seen in other cell types (17,34). Doc2b is known to require very little calcium to translocate in neurons (35). Skeletal muscle may have baseline calcium already high enough to translocate Doc2b under resting conditions. Under such conditions, Doc2b can be considered constitutively active (35), which can explain the strong effects in the Doc2b−/− mice observed here, relative to effects previously observed in brain (23). In 3T3-L1 adipocytes, Doc2b is reported to bind to syntaxin 4 only under high calcium buffer conditions (17). Therefore, we simulated those calcium conditions to investigate the physiological occurrence/relevance of this putative Doc2b–syntaxin 4 complex in primary skeletal muscle. However, regardless of calcium levels in skeletal muscle extracts, Doc2b failed to coprecipitate in anti–syntaxin 4 immunoprecipitation reactions, suggesting that such an interaction might not be a dominant factor in primary cells.

Concerning the mechanism of Doc2b actions in both insulin granule and GLUT4 vesicle exocytosis, several possibilities might be considered. One possibility is that Doc2b serves as a platform for transient interactions with Munc18 and syntaxin. According to this “switch hypothesis” model, derived from β-cell studies (16,18), Munc18c becomes tyrosine phosphorylated in response to a stimulus, dissociates from syntaxin 4, and switches its binding preference to Doc2b. Doc2b’s sequestration of Munc18c would facilitate
FIG. 5. Insulin-dependent, but calcium-independent, Doc2b-Munc18c association in mouse skeletal muscle. The impact of insulin stimulation and/or calcium addition to lysis buffer upon association of Munc18c with Doc2b (A) or VAMP2, SNAP23, and syntaxin 4 (B) was assessed by reciprocal coimmunoprecipitation reactions using hindlimb skeletal muscle extracts. Reactions were processed in parallel from the same starting hindlimb muscle extracts from WT mice injected with vehicle (saline) or insulin (10 units/kg body weight) for 5 min in lysis buffers supplemented with either 2 mmol/L EDTA or 1 mmol/L CaCl2. Immunoprecipitated proteins were resolved on 10–12% SDS-PAGE for immunodetection of Munc18c, Doc2b, syntaxin 4, SNAP23, and VAMP2. Equivalent abundance of proteins in the corresponding starting lysates was confirmed by immunoblot (Lysate).

C: Calcium addition to lysis buffer does not facilitate Doc2b coimmunoprecipitation with anti-syntaxin 4 from skeletal muscle extracts. Muscle extracts used in A and B were subjected to anti-syntaxin 4 immunoprecipitation for immunodetection of Doc2b. SNAP23 binding to syntaxin 4 validated the immunoprecipitation reactions. Control IgG and lysates from Doc2b−/− mice were used in separate reactions to control for nonspecific banding occurring with the Doc2b antibody. D: Evaluation of Doc2b protein recruitment to the PM fraction in response to insulin. P2 fraction extracts prepared from saline or insulin-stimulated WT mice were subjected to SDS-PAGE as described in Fig. 4B for immunodetection of Doc2b and syntaxin 4 (Syn4). Data are representative of three independent sets of homogenates or fractions. IB, immunoblot.

FIG. 6. Munc18c–syntaxin 4 binding is increased in Doc2b−/− mouse skeletal muscle. Male, 4–6-month-old Doc2b+/+ and Doc2b−/− littermate mice were injected with vehicle (saline) or insulin (10 units/kg body weight) for 5 min, hindlimb muscles were excised, and detergent extracts were prepared for use in anti-Munc18c (A) or anti-syntaxin 4 (Syn4) (B) immunoprecipitation reactions. Immunoprecipitated proteins were resolved on 10% SDS-PAGE for immunodetection of Doc2b and syntaxin 4 (Syn4). Equivalent abundance of proteins in the corresponding starting lysates was confirmed by immunoblot (Lysate). C: Sarcolemmal/transverse tubule membrane fractions (P2) were used in anti-syntaxin 4 immunoprecipitation reactions to capture binary and ternary SNARE complexes, composed of VAMP2 and SNAP23, and syntaxin 4–Munc18c complexes, all resolved on 12% SDS-PAGE for immunoblotting. Optical density quantitation of three independent pairs of Doc2b+/+ and Doc2b−/− muscle fractions is shown in the bar graphs. *P < 0.05 compared with insulin-stimulated WT. IB, immunoblot; IP, immunoprecipitation.
DISRUPTED GLUCOSE HOMEOSTASIS IN Doc2b−/− MICE

syntaxin 4’s participation in SNARE complexes to promote vesicle fusion. Such a model is consistent with 1) the insulin-stimulated association of Doc2b with Munc18c in skeletal muscle and 2) the strong increase in Munc18c binding to syntxin 4, concurrent with the reduced binding of VAMP2 and SNAP23 to syntxin 4 in sarcolemmal/transverse tubule muscle membrane fractions, indicative of attenuated SNARE complex formation in the absence of Doc2b (Fig. 6C). Alternatively, Doc2b may facilitate fusion via a different or additional mechanism, by partially inserting into the PM upon calcium binding, and induce membrane deformations that assist merging vesicle and PM. This property contributes to the exceptional in vitro fusogenic properties of Doc2b relative to all other C2-domain proteins studied (23).

Although the disease phenotype of the Doc2b−/− knock-out mice was almost as severe as that of the Doc2b−/− mice, interpreting the relative contribution of insulin secretory defects versus insulin resistance is complex. For example, insulin content in the serum after the acute glucose challenge trended toward a decrease (P = 0.08, n = 6), intermediate between that of the WT and Doc2b−/− mice, but did not reach statistical significance. However, since serum insulin content is not an absolute readout of insulin secretion but rather a net readout of pancreatic insulin release, hepatic insulin clearance, and insulin utilization by other tissues, use of the hyperglycemic clamp approach will be required for full assessment. Also noteworthy was that the initial drop (15–30 min) in blood glucose in the ITT in the Doc2b−/− mice was similar to that of WT mice, seemingly counter to the blunted glucose uptake into the EDL of the Doc2b−/− mice. However, the glucose uptake assay was performed in vitro using excised muscle, whereas the ITT is performed in vivo. In vivo, the insulin bolus will initiate a decrease in hepatic glucose output. Given that hepatic insulin signaling in the Doc2b−/− mice was normal, it would seem to be a likely contributor to the initial blood glucose drop.

CONCLUSIONS

The data presented here demonstrate a key role for Doc2b in multiple exocytotic processes relevant to the maintenance of whole-body glucose homeostasis, including insulin secretion and peripheral glucose clearance. We propose that Doc2b engages in stimulus-dependent association with Munc18c in skeletal muscle similar to that in β-cells; this implicates the mechanisms to be highly conserved, albeit the stimuli are cell-type specific. Furthermore, our data demonstrating the need for Doc2b in first-phase insulin release suggest that it may also participate as a scaffolding platform for Munc18-1 binding in the β-cell. Novel reagents based upon Doc2b may carry promise as dual insulin-sensitizing/insulin secretion enhancement approaches to combating a combinatorial disease like type 2 diabetes.

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L.R., E.O., S.M.Y., and M.A.K. researched data, contributed to discussion, and reviewed and edited the manuscript. J.T.B. performed the glucose transport assay and contributed to discussion. A.J.G. and M.V. provided the Doc2b−/− mice, reagents, and protocols and edited the manuscript. D.C.T. contributed to discussion and wrote, reviewed, and edited the manuscript. D.C.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this study were presented at the 71st Scientific Sessions of the American Diabetes Association, San Diego, California, 24–28 June 2011.

The Vanderbilt Mouse Phenotyping Core Facility quantified measurements of serum NEFA.

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